Nitrate Contamination of Drinking Water: Relationship with *HPRT* Variant Frequency in Lymphocyte DNA and Urinary Excretion of *N*-Nitrosamines

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We studied peripheral lymphocyte *HPRT* variant frequency and endogenous nitrosation in human populations exposed to various nitrate levels in their drinking water. Four test populations of women volunteers were compared. Low and medium tap water nitrate exposure groups (14 and 21 subjects) were using public water supplies with nitrate levels of 0.02 and 17.5 mg/l, respectively. Medium and high well water nitrate exposure groups (6 and 9 subjects) were using private water wells with mean nitrate levels of 25 and 135 mg/l, respectively. Higher nitrate intake by drinking water consumption resulted in a dose-dependent increase in 24-hr urinary nitrate excretion and in increased salivary nitrate and nitrite levels. The mean log variant frequency of peripheral lymphocytes was significantly higher in the medium well water exposure group than in the low and medium tap water exposure groups. An inverse correlation between peripheral lymphocyte labeling index and nitrate concentration of drinking water was observed. Analysis of *N*-nitrosamine in the urine of 22 subjects by gas chromatography–mass spectrometry revealed the presence of *N*-nitrosopyrrolidine in 18 subjects. Analysis of the mutagenicity of well water samples showed that a small number of the well water samples were mutagenic in the Ames *Salmonella typhimurium* test after concentration over XAD-2 resin. In conclusion, consumption of drinking water, especially well water, with high nitrate levels can imply a genotoxic risk for humans as indicated by increased *HPRT* variant frequencies and by endogenous formation of carcinogenic *N*-nitroso compounds from nitrate-derived nitrite. *Key words*: drinking water, *HPRT*, nitrate, nitrosamines. *Environ Health Perspect* 104:522–528 (1996)

The major human health risk associated with exposure to nitrate is considered to be methemoglobinemia due to endogenous conversion of nitrate to nitrite. The World Health Organization drinking water guideline value for nitrate has been set at a value of 45 mg/l, which is suitable to prevent development of methemoglobinemia (*1*). In the European Union, the maximum admissible nitrate level in drinking water has been set at 50 mg/l (*2*). Two to five percent of the drinking water sources in the European Union countries have nitrate concentrations exceeding 50 mg/l, which means that several million people in Europe are receiving a water supply that exceeds this limit (*3*).

Nitrite derived from nitrate may react *in vivo* with amines and amides to form *N*-nitroso compounds, which may have carcinogenic properties. However, the drinking water standard of nitrate has not been based on this possible formation of *N*-nitroso compounds. A high nitrite intake has been positively associated with stomach cancer risk (*4*). A consistent association has been observed between nitrate intake and the rate of endogenous nitrosation of proline, which has been used as a measure of the potential for endogenous formation of carcinogenic *N*-nitroso compounds (NPRO test) (*5,6*). In a number of studies, the NPRO test has been successfully used to compare the potential for endogenous nitrosation in populations with different rates of gastric cancer (*7–12*). NPRO excretion in all studies was highest in the area with the highest rate of gastric cancer, but only significantly so in a Chinese and a Japanese study (*7,8*). In contrast, case–control studies using food-frequency questionnaires tend to show a protective effect of the estimated nitrate intake on gastric cancer risk (*13–15*). Most likely this is due to the known strong protective effect of fruits and nitrate-containing vegetables, in particular when containing high levels of vitamin C, on the risk of gastric cancer. When drinking water with no or little nitrate (e.g., 0–5 mg/l) is consumed, this source of nitrate intake is almost negligible. However, when the nitrate concentration of drinking water is higher (e.g., 50–100 mg/l), 65–80% of the total nitrate intake and 50–65% of the total nitrate exposure may result from drinking water consumption (taking also the endogenous formation of nitrate into account).

In Colombia and Italy, high levels of nitrate in well water were associated with an increased risk of gastric cancer (*16,17*). In a cross-sectional study in an area with a high incidence of gastric cancer in northeastern China, an association between high levels of nitrate in drinking water supplies and neoplastic changes in the stomach was observed (*18*). The type of drinking water supply has been found to be a risk factor for stomach cancer. In particular, the use of private water sources, especially well water, has been associated with stomach cancer risk (*4*). Possible etiological mechanisms include increased nitrate concentration without concurrent increases in vitamin C intake, and the presence of particular microorganisms. Gao (*19,20*) and Chen (*21*) assume that mutagenic substances in well water are the etiological factor for stomach cancer in China. In addition to the role of *in vivo* formation of *N*-nitroso compounds in gastric carcinogenesis (*22,23*), a role in the etiology of cancer of the esophagus (*22,24*) and of the nasopharynx (*22,25*) has been suggested.

The Province of Limburg in The Netherlands is an area with a high nitrate burden due to agricultural application of animal manure. To examine the possible genotoxic risk of consumption of drinking water with high nitrate levels in this area, we have previously studied the occurrence of peripheral lymphocyte sister chromatid exchanges in populations exposed to different nitrate concentrations in drinking water, including a subpopulation using well water with nitrate concentrations in the range of 50–300 mg/l (*26*). In this study, no relationship was observed between sister chromatid exchange frequencies and increased exposure to nitrate due to consumption of drinking water with high nitrate concentrations. In a subsequent study performed in the same populations, we studied the effect of consumption of drinking water with high nitrate levels on the thyroid. A dose-dependent difference in the size of the thyroid was observed between low and medium versus high nitrate exposure groups, with enlarged thyroid volume at nitrate levels exceeding 50 mg/l (*27*).

We have continued to investigate the applicability of genetic biomarkers for cancer risk assessment of nitrate contamination of drinking water. The present study describes the use of the *HPRT* (hypoxanthine-guanine phosphoribosyltransferase) variant frequency (VF) test in lymphocytes as an index for

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genetic risk in human populations exposed to different nitrate levels in drinking water. This is one of the few methods available for monitoring mutagenicity in human populations exposed to environmental mutagens (*28*). Furthermore, we investigated endogenous nitrosation as a consequence of increased intake of nitrate by monitoring the urinary excretion of *N*-nitrosamines. The mutagenicity of well water samples with high nitrate levels was also investigated.

Materials and Methods

In four test populations with different levels of drinking water nitrate concentrations, we studied peripheral lymphocyte variant frequencies as an index for mutations in relation to endogenous nitrosation. The test populations consisted of healthy women volunteers who were free of diseases, did not use medications, were not pregnant, and had no outdoor jobs. Two test populations consisted of a "low tap water" exposure group (n = 14, group A) and a "medium tap water" exposure group (n = 21, group B) living in municipalities with drinking water supplies containing 0.02 mg/l and 17.5 mg/l nitrate, respectively; the nitrate level of drinking water of the latter group had been lowered 1 year before the onset of this study from 32.0 mg/l to 17.5 mg/l (*26*). Two other test populations consisted of a "medium well water" (n = 6, group C) and "high well water" (n = 9, group D) exposure group consisting of subjects who used private wells for their drinking water supply with nitrate levels below 50 mg/l (group C, mean nitrate level 25 ± 15 mg/l, range 8–43 mg/l) and above 50 mg/l (group D, mean nitrate level 135 ± 76 mg/l, range 55–270 mg/l), respectively. The age distribution of the different exposure groups was 41 ± 9, 42 ± 8, 38 ± 6, and 45 ± 10 years for groups A, B, C, and D, respectively. No significant differences in age were found between the groups. The distribution of smokers and nonsmokers of the four groups is shown in the legend to Table 1.

The subjects agreed to answer a questionnaire on food consumption, with emphasis on food items that substantially contribute to nitrate uptake, and lifestyle habits, as well as to donate saliva, 24-hr urine, and venous blood samples. Oral intake of nitrate was calculated from the intake of food constituents using data on nitrate content from Dutch food quality monitoring programs and data from the analyses we performed on nitrate concentrations of the public water supplies and private water wells.

During home visits to the subjects, we obtained 5-ml saliva and 10-ml venous blood samples. Within 24 hr before the visit, urine was collected by the subjects. Water samples were collected for analysis of nitrate from the private water wells and from the drinking water supplies of the low and medium tap water exposure groups.

The nitrate and nitrite concentrations of saliva and urine samples and the nitrate concentration of water samples were determined as previously described (*26*). Urinary nitrate excretion was calculated as milligrams nitrate per 24 hr. The frequency of 6-thioguanine-resistant (TGr) human peripheral lymphocytes (*HPRT* VF) in the blood samples of the subjects was determined as previously described (*28*).

The mutagenicity of 12 well water samples with nitrate levels in the range of 50–300 mg/l was determined as follows (*29,30*). We applied 4.5 l well water to a column packed with XAD-2 resin, eluted the column with 500 ml distilled water and dried under nitrogen. To elute components with different polarity absorbed to the XAD-2 resin, the column was eluted with 45 ml acetone or 4.5 ml dimethylsulfoxide (DMSO). The acetone eluents were concentrated to a volume of about 10 ml on a rotary evaporator and to dryness under nitrogen, and the residues were dissolved in 1.5 ml DMSO. The DMSO fractions were tested for mutagenicity in the Ames test using *Salmonella typhimurium* strains TA98 and TA104, in presence and absence of S9 fraction. Strain TA98 was selected because it has been used in most of the studies on mutagenicity of drinking water prepared from groundwater. Strains TA98 and TA100 have been reported to be the most sensitive strains for detection of mutagenicity in drinking water (*30*). Strain TA104 was selected because it was the most sensitive strain in a pilot experiment on mutagenicity using river water from the area where the investigations were performed (the Jeker River). Strain TA104 is sensitive to oxidative mutagens (*30*). With this procedure, we tested 3000-fold and 1000-fold concentrated samples (acetone and DMSO eluents, respectively). Portions of well water samples applied to the XAD-2 column were also acidified to pH 2 with 4 N HCl to suppress ionization of acidic components (*30*); the samples were applied again to the XAD-2 column for absorption of these components to the resin, the column was eluted with acetone or DMSO, and the eluents were tested for mutagenicity in the Ames test. Alternatively, 500-ml well water samples were concentrated to a volume of 50-ml on a rotary evaporator and tested for mutagenicity to detect relatively polar mutagenic compounds that do not absorb to XAD-2 resin. Thus, each of the 12 well water samples was tested 5 times for mutagenicity (acetone and DMSO eluents of neutral and acidic well water samples applied to XAD-2 resin and 10-fold concentrated well water samples; see Table 3).

To examine the urinary excretion of *N*-nitrosamines, we set up an assay to determine eight volatile *N*-nitrosamines by GC–MS. The compounds for which the urine samples were tested are listed in Table 2. The *N*-nitrosamines in the 24-hr urine samples were determined by the following pro-

Table 1. Mean nitrate intake parameters, nitrate biomonitoring parameters, variant frequencies (VF), and labeling indices of the four nitrate exposure groups (± SDs)[a]

	A: low TW nitrate	B: medium TW nitrate	p A:B	C: medium WW nitrate	p A:C	p B:C	D: high WW nitrate	p A:D	p B:D	p C:D
Nitrate via drinking water (mg/24 hr)	0	25 ± 8	0.0001	34 ± 25	0.0001	ns	132 ± 111	0.0001	0.0001	0.01
Nitrate via food (mg/24 hr)	145 ± 77	173 ± 11	ns	126 ± 25	ns	ns	99 ± 30	ns	0.03	ns
Total nitrate intake (mg/24 hr)	145 ± 77	198 ± 114	ns	160 ± 21	ns	ns	231 ± 122	0.04	ns	ns
Nitrate excretion (mg/24 hr)	41 ± 20	67 ± 42	0.02	84 ± 27	0.003	ns	136 ± 94	0.005	0.04	ns
Nitrate in saliva (µg/ml)	21 ± 11	36 ± 34	ns	22 ± 25	ns	ns	47 ± 47	ns	ns	ns
Nitrite in saliva (µg/ml)	0.6 ± 0.5	0.9 ± 0.7	ns	0.7 ± 0.5	ns	ns	2.0 ± 2.1	0.02	0.03	0.04
VF of TG^r lymphocytes ($\times 10^{-6}$)	44 ± 60	24 ± 24	ns	264 ± 389	ns	0.01	148 ± 366	ns	ns	ns
log (VF $\times 10^{-6}$)	1.2 ± 0.7	1.2 ± 0.4	ns	2.0 ± 0.8	0.03	0.002	1.4 ± 0.8	ns	ns	ns
Labeling index	0.15 ± 0.08	0.19 ± 0.05	ns	0.16 ± 0.09	ns	ns	0.13 ± 0.04	ns	0.006	ns

Abbreviations: TW, tap water; WW, well water; ns, nonsignificant; VF, variant frequency; TG^r, thioquanine resistant.
[a]The age distribution and smoking behavior of the 4 subgroups were: group A: 41 ± 9 years (4 smokers/10 nonsmokers); group B: 42 ± 8 (7/14); group C: 38 ± 6 (1/5); group D: 45 ± 10 (2/7).

cedure. We added 1 ml borate buffer, pH 10, to 20-ml urine samples and extracted the resulting solution with 2 ml dichloromethane (*31*). The dichloromethane layer was separated, and 1 µl was introduced into the capillary GC-MS system, consisting of an HP 5890 series II gas chromatograph (Hewlett-Packard, Avondale, Pennsylvania) and a Jeol SX102A sector field mass spectrometer (Jeol Ltd., Tokyo). The column used was a 25-m fused silica SGE BPX5 column, i.d. 0.32 mm, and film thickness 0.25 µ. Helium was used as the carrier gas at a flow rate of 1 ml/min. Ions were generated by electron ionization at 70 eV electron energy. Optimum sensitivity was obtained by measuring the M+· ion intensity in the selected ion monitoring (SIM) mode. The m/z values of these ions are given in Table 2. With this procedure, detection limits were about 0.2 pg/µl dichloromethane solution introduced, corresponding to 20 pg/ml urine, for each of the *N*-nitrosamines investigated. Calibration was performed using a nitrosamine mixture for EPA method 8270 (Aldrich, Bornen, Belgium). For some samples, high-resolution single-ion monitoring (HR-SIM) mass spectrometry was applied to confirm the elemental composition of the ions detected using low-resolution SIM (see Table 4).

Statistical analyses of differences between groups with respect to nitrate intake via drinking water and food, nitrate and nitrite levels in saliva, urinary nitrate excretion, urinary *N*-nitrosamine excretion, *HPRT* VF, and labeling index were performed by the ANOVA test and the nonparametric Mann-Whitney *U*-test for parameters without linear distribution. Linear, multiple, and stepwise regression was used to investigate the relationship between urinary nitrate excretion, urinary *N*-nitrosamine excretion, and salivary nitrate and nitrite concentrations versus nitrate intake from drinking water, food, and total intake; between urinary *N*-nitrosamine excretion and urinary nitrate excretion, salivary nitrate and nitrite concentrations, and smoking behavior; between *HPRT* VF and nitrate and *N*-nitrosamine excretion, salivary nitrate and nitrite concentrations, age and smoking behavior, and labeling index of control cultures; and between labeling index and nitrate concentration of drinking water. Because of the nonlinearity of the VFs, logarithmically transformed VFs were used in the regression analyses. As an inverse correlation between log VF and the labeling index of control cultures has been reported (*28*), the labeling index was included in the regression analyses. Mutagenicity of well water samples was determined by least significant difference method (*32*). Linear regression analysis was performed between the number of mutagenic samples per analyzed well water and the nitrate or nitrite concentration of the particular well water and multiple regression analysis between the number of revertants/l well water and the nitrate and nitrite concentrations of well water.

Table 2. *N*-Nitroso compounds for which the urine samples were screened

		M+· ion
1.	*N*-Nitrosodimethylamine	74.05
2.	*N*-Nitrosomethylethylamine	88.06
3.	*N*-Nitrosodiethylamine	102.08
4.	*N*-Nitrosopyrrolidine	100.06
5.	*N*-Nitrosopiperidine	114.08
6.	*N*-Nitrosomorpholine	116.06
7.	*N*-Nitrosodi-*n*-propylamine	130.11
8.	*N*-Nitrosodi-*n*-butylamine	158.14

Results

The mean calculated daily nitrate intake from drinking water, food, and total intake from drinking water and food of the four test populations are shown in Table 1. Significant differences were observed in drinking water nitrate intake among any combination of two groups, except there were no differences between groups B and C. No significant differences in nitrate intake from food were found between the nitrate exposure groups, except for a significantly lower intake in group D as compared with group B. The analysis of total nitrate intake revealed a significant difference in intake between groups A and D. The mean 24-hr urinary nitrate excretions and salivary nitrate and nitrite concentrations are shown in Table 1. Significant differences in urinary nitrate excretion were observed between group A and groups B, C, or D and between groups B and D. No significant differences in salivary nitrate concentrations between any two exposure groups were observed, but the analysis of salivary nitrite concentrations revealed significant differences between groups A and D, between groups B and D, and between groups C and D. Linear regression analysis applied to the total number of subjects (n = 50) revealed a significant correlation between salivary nitrate levels and total nitrate intake (p = 0.002; Fig. 1) and between both 24-hr urinary nitrate excretion and salivary nitrite concentration versus drinking water nitrate concentration (p = 0.0001 and p = 0.004, respectively). Stepwise regression analysis between 24-hr urinary nitrate excretion, salivary nitrate levels, and salivary nitrite levels versus drinking water nitrate doses, nitrate doses from food, and nitrate doses from total intake revealed the most significant correlation between salivary nitrate concentration and nitrate doses from total intake. Thus, the analyses of the differences between the exposure groups and the regression analyses show that drinking water nitrate contamination causes a dose-dependent increase in 24-hr urinary nitrate excretion, as well as in salivary nitrate and nitrite concentrations.

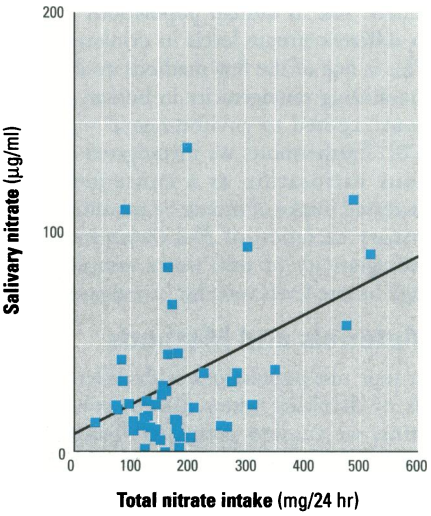

Figure 1. Linear regression analysis between total nitrate intake and salivary nitrate levels for all subjects (n = 50; p = 0.002, r^2 = 0.19).

The numbers of mutagenic well water samples found using different analytical methods are shown in Table 3. Of the 60 samples tested for mutagenic activity (12 well water samples tested using 5 concentration procedures), 11 samples were mutagenic. The largest number of mutagenic samples was found using acetone to elute the XAD-2 resin (6 samples); 2 samples were mutagenic using DMSO as the eluent, and 3 samples were mutagenic using the directly 10-fold concentrated well water samples. This pattern suggests the presence of both polar and apolar mutagenic substances in the well water. In the linear and multiple regression analyses, no significant correlations were found between the total number of mutagenic samples per analyzed well water or the number of revertants per liter well water and the nitrate and/or nitrite concentrations of the particular well water samples. Also, no correlations were observed when a distinction was made between the different strains used and the application of S9 fraction. It has been recommended in mutagenicity testing to evaluate mutagenic potential based on results obtained not only with strains TA98 and TA100, but also with strains TA102 and TA104, covering a broad spectrum of potentially mutagenic compounds (*30*). However, using both strains TA98 and TA104 did not result in the detection of a large number of mutagens in

Table 3. Number of mutagenic samples from 12 well water samples, each tested using 5 different concentration procedures

Absorption to XAD-2 resin	*Salmonella typhimurium* strain TA98 +S9	TA98 -S9	TA104 +S9	TA104 -S9
Neutral fraction				
Acetone eluent	0	0	3	0
DMSO eluent	0	0	0	0
Acidic fraction				
Acetone eluent	0	1	1	1
DMSO eluent	0	0	1	1
Total sample evaporated	0	0	2	1

DMSO, dimethylsulfoxide.

well water samples.

The presence of *N*-nitrosamines in urine samples was examined in 11 subjects of the tap water exposure groups A and B and in 11 subjects of the well water exposure groups C and D. The results of the analyses of the urine samples are shown in Table 4. The *N*-nitroso compounds 2, 6, 7, and 8 (see Table 2) were not detected in any of the urine samples. The *N*-nitroso compounds 1, 3, and 5 were detected in some of the urine samples, and *N*-nitroso compound 4 was detected in most of the urine samples. Accurate mass measurement of *N*-nitroso compound 4 showed the correct elemental composition for *N*-nitrosopyrrolidine. No significant differences in the mean 24-hr urinary excretions of these *N*-nitroso compounds were found between the tap water exposure groups A and B and the well water exposure groups C and D (146 ± 173 ng and 160 ± 95 ng, respectively). Linear, multiple, and stepwise regression analysis of the data of the 22 subjects on which measurements of *N*-nitroso compounds were performed revealed a significant correlation between 24-hr urinary excretion of *N*-nitrosopyrrolidine (compound 4) and 24-hr urinary nitrate excretion ($p = 0.02$, Fig. 2).

The mean VFs of the blood samples of the four different nitrate exposure groups are shown in Table 1. A significant difference in mean log VF was observed between groups A and C and between groups B and C. However, in the well water exposure groups C and D, two extremely high VFs were observed (1045×10^{-6} and 1121×10^{-6}, respectively), which can be regarded as outliers. The mean VFs of groups C and D without these high values were $108 \pm 76 \times 10^{-6}$ and $26 \pm 35 \times 10^{-6}$, respectively. When the analysis was performed without the two outliers, significant differences in mean log VF were still observed between groups A and C and between groups B and C, the mean log VF of group C being significantly higher than the mean log VF of groups A and B. Linear regression analysis between log VF and nitrate intake, nitrate excretion, *N*-nitrosamine excretion, salivary nitrate and nitrite levels, age and smoking behavior, and labeling index of control cultures showed no significant correlation between log VF and any single parameter. The correlation between log VF and labeling index was negative, but not significant ($p = 0.15$). Multiple regression analysis showed a significant correlation between log VF versus 24-hr urinary nitrate excretion, salivary nitrite levels, and labeling index (whole model, $p = 0.03$; 24-hr nitrate excretion, $p = 0.02$; salivary nitrite levels, $p = 0.03$). In this model, the β coefficient was positive for urinary nitrate excretion and negative for salivary nitrite levels. Linear regression analysis between labeling index and the nitrate concentration of the drinking water

Table 4. Urinary excretion of *N*-nitrosamines with M+· of 74 (compound 1), 102 (compound 3), 100 (compound 4) and 114 (compound 5) of subjects exposed to different nitrate drinking water concentrations (ng/24 hr)[a]

Subject no.	Exposure group	Nitrate in drinking water (mg/l)	Nitrate excretion (mg/24 hr)	M+· 74	102	100[b]	114	Sum
22	D	270	268	0	61	81	0	142
21	D	217	260	0	0	252	0	252
7	D	188	14	0	0	0	68	68
49	D	120	191	0	57	184	0	241
17	D	96	62	0	0	75	0	75
37	D	65	119	0	0	86	232	318
42	D	61	94	0	0	39	0	39
8	D	55	79	0	0	83	64	147
23	C	37	117	0	0	43	77	120
34	C	30	116	0	0	272	0	272
24	C	19	63	0	0	90	0	90
12	B	17.5	23	0	0	16	0	16
3	B	17.5	69	0	0	0	0	0
6	B	17.5	24	0	0	161	0	161
59	B	17.5	70	49	0	171	0	220
28	B	17.5	193	0	177	132	0	309
26	B	17.5	113	0	0	61	0	61
2	A	0	28	0	0	0	0	0
1	A	0	40	0	0	0	0	0
33	A	0	70	0	0	58	122	180
69	A	0	94	200	0	87	279	566
65	A	0	25	0	0	100	0	100

[a]Levels of compounds 2, 6, 7, and 8 were below the detection limit for all samples; see text for description of exposure groups; Table 2 for list of compounds.
[b]Elemental composition $C_4H_8N_2O$ confirmed by high-resolution–single ion monitoring mass spectrometry.

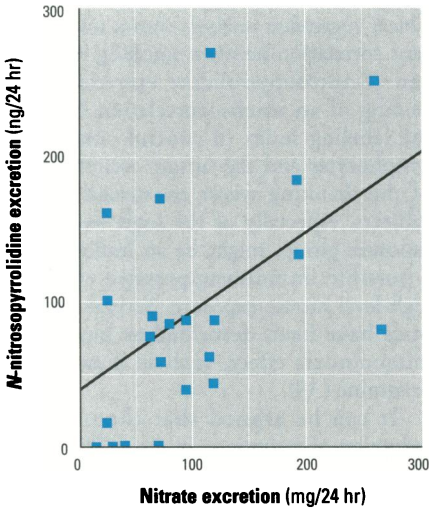

Figure 2. Linear regression analysis between 24-hr urinary excretion of *N*-nitrosopyrrolidine and 24-hr urinary nitrate excretion for 22 subjects ($p = 0.02$, $r^2 = 0.26$).

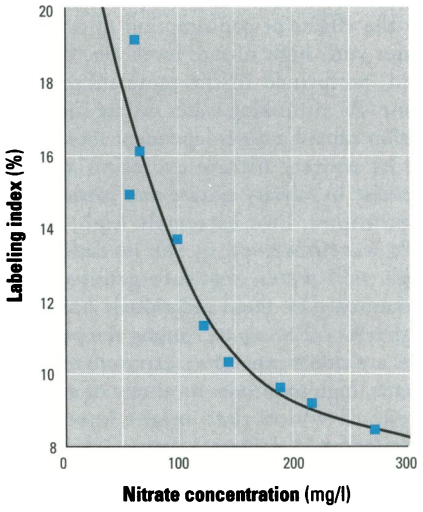

Figure 3. Polynomial regression analysis between nitrate concentration of drinking water and labeling index of control lymphocyte cultures for the subjects of the high well-water exposure group ($n = 9$, $r^2 = 0.88$).

used by the subjects showed a significant inverse correlation (p = 0.03, r^2 = 0.1). Figure 3 shows that this correlation is mainly determined by the strong inverse correlation between labeling index and the nitrate concentration of the drinking water of the high well water exposure group (r^2 = 0.88).

Discussion

In view of the reported increase in gastric cancer risk due to consumption of well water with high levels of nitrate, we thought it important to study the applicability of genetic biomarkers as early indicators of carcinogenic or mutagenic effects. In a first study in subpopulations exposed to different nitrate concentrations in their drinking water, including a subpopulation using well water with nitrate concentrations in the range of 50–300 mg/l, no increase in the frequency of peripheral lymphocyte sister chromatid exchanges was observed (*26*). In the present study, we analyzed the *HPRT* VF in relation to endogenous nitrosation by monitoring the urinary excretion of *N*-nitrosamines in order to study the risks associated with nitrate exposure due to consumption of drinking water with high nitrate levels.

In accordance with our previous study on the effects of consumption of drinking water with high nitrate levels on the thyroid, performed in the same subpopulations (*27*), drinking water nitrate contamination caused a dose-dependent increase in 24-hr urinary nitrate excretion and an increase in salivary nitrate and nitrite concentrations. Two extremely high *HPRT* VFs were observed in the medium and high well-water exposure groups. It is unknown why these individuals had such high levels of mutants. Among the possibilities are clonal expansion, exposure to other agents, high sensitivity to nitrate or another agent to which they might have been exposed, and defective repair. However, since the VFs we found in this study were about a factor of 10–100 higher than the VFs we normally obtain, these values could be due to false positive results (possibly due to the use of purine-rich fetal calf serum). When the extremely high values were omitted from the analysis, the mean log VF was still significantly higher in the medium well-water exposure group with nitrate levels <50 mg/l than in the low and medium tap-water exposure groups.

Using a multiple regression model, a significant correlation was found between log VF and urinary nitrate excretions and salivary nitrite levels, but this correlation was only positive for urinary nitrate excretion. In humans environmentally or occupationally exposed to ionizing radiation, cytostatic agents, ethylene oxide, vinyl chloride, and other mutagens, increased VFs have been reported (*28*). However, this study is the first to report an increase in peripheral lymphocyte *HPRT* VF due to high-level nitrate exposure. Effects of smoking behavior and age on VFs of TGr lymphocytes have also been described (*28*). In our study, the multiple regression analysis revealed no significant correlations between log VF and age or smoking behavior. The exposures due to smoking are lower among the subjects using well water (3 smokers out of 15 subjects using well water versus 11 smokers out of 35 subjects using tap water). Of the three smokers using well water, two had the highest VFs within their groups and 1 subject had a very low VF. The smokers in the tap water exposure groups showed no increased VFs within their groups. A further analysis of the individual data of the two smoking subjects with high VFs in the well water exposure groups showed that one subject had the highest nitrate concentration in drinking water (270 mg/l) and one subject had the highest *N*-nitrosopyrrolidine excretion (272 ng/24 hr). Thus, smoking probably does not account for the observed increase in VFs in these two subjects. Linear regression analysis shows no significant correlation between smoking behavior and *N*-nitrosopyrrolidine excretion. The finding of an inverse correlation between the labeling index of control cultures of lymphocytes and the nitrate concentration of the drinking water consumed by the subjects, especially of the high well-water exposure group, might be an indication of a possible immunosuppressive effect of high-level nitrate exposure. Several xenobiotics have been described as having an immunotoxic effect, such as dimethylnitrosamine (*33*).

It can be argued that the possible induction of mutations in the *HPRT* locus in peripheral lymphocytes during high nitrate exposure is caused by nitrite and/or *N*-nitrosamines formed from ingested nitrate, as nitrate per se is not genotoxic, but the mutagenicity of nitrite and in particular the mutagenicity and carcinogenicity of *N*-nitroso compounds have been reported (*3*). Recently, the induction of *HPRT* gene mutations in skin fibroblasts of rats exposed *in vivo* to *N*-nitrosamines has been described (*34*). Exposure to drinking water containing a mixture of pesticides and ammonium nitrate that simulated contaminated groundwater in California was shown to cause genetic damage in rodents (*35*). Since some of the well water samples in our study had mutagenic activity, the possibility has to be considered that an increase in *HPRT* VF is caused by other components of well water rather than by the high nitrate content. A high nitrate content of well water might be indicative of the presence of other contaminants, but no correlation was observed between mutagenicity of well water and nitrate and/or nitrite content of well water, and only a small number of well water samples was mutagenic. Because a correlation was observed between nitrate exposure and increased VF, the increase in VF is probably not caused by the presence of mutagens in the well water. Also, the subjects with the highest VFs did not use well water that was found to be mutagenic.

The GC–MS assay used for the analysis of *N*-nitrosamines enables the detection of eight volatile *N*-nitrosamines; several of these have been proven to be carcinogenic (*N*-nitrosodimethylamine, -diethylamine, -piperidine, and -pyrrolidine). Analysis of the urine samples of the subjects revealed the presence of carcinogenic *N*-nitroso compounds. In a small number of the analyzed urine samples, the presence of *N*-nitrosodimethylamine, *N*-nitrosodiethylamine, and *N*-nitrosopiperidine was observed (in 2, 3, and 6 of the 22 analyzed samples, respectively). In most of the samples (18 out of 22), *N*-nitrosopyr-rolidine was detected. Although no difference in mean 24-hr excretion of *N*-nitrosamines was observed between the tap water and well water exposure groups, a correlation between 24-hr excretion of *N*-nitrosopyrrolidine and 24-hr nitrate excretion for all subjects was observed. Linear regression analysis showed no significant correlation between smoking behavior and *N*-nitrosopyrrolidine excretion.

Spiegelhalder et al. (*36*) found that *N*-nitrosodimethylamine (NDMA) could only be detected in the urine during administration of aminopyrine with simultaneous intake of ethanol, due to inhibition of the relevant metabolizing cytochrome P450 isoenzyme that rapidly metabolizes NDMA. In our study, no increasing effect of alcohol consumption on urinary nitrosamine excretion was observed. Increased urinary nitrosamine excretion has been observed in paraplegic and bilharzia bladder patients (*37,38*). In these patients a bacterially mediated formation of *N*-nitroso compounds occurs in the urinary tracts due to a high conversion of nitrate into nitrite. There was no nitrite detectable in the urine samples of the subjects in our study, thus the *N*-nitrosamines we detected are probably not produced by bacteria in the urinary tracts.

Garland et al. (*39*) observed the urinary excretion of NDMA and nitrosoproline in

healthy volunteers by gas chromatography–high resolution mass spectrometry analysis of urine samples. It could be argued that two of the *N*-nitrosamines detected in urine in our study, *N*-nitrosopyrrolidine and NDMA, might have been formed by thermal decarboxylation of *N*-nitrosoproline and *N*-nitrososarcosine during the GC–MS analysis. However, since the urine samples were extracted under alkaline conditions, this possibility can be ruled out. The presence of volatile *N*-nitrosamines in the stomachs of volunteers who were given meals with different nitrate contents (derived from vegetables) and containing meat, eggs, or fish has been described (*40*). There is evidence that volatile *N*-nitrosamines (e.g., NDMA, *N*-nitrosodiethylamine, *N*-nitrosopiperidine, and *N*-nitrosopyrrolidine) are absorbed rapidly from the duodenum and are subsequently carried by the portal circulation to the liver (*41*). Extensive metabolism of the volatile *N*-nitrosamines occurs; Spiegelhalder et al. (*42*) found that <1% of a dose of NDMA was excreted unchanged in the urine of rats. Thus, the presence of these volatile *N*-nitrosamines in the urine of subjects exposed to high nitrate concentrations suggests that a much larger amount of these nitrosamines is being formed in the body. These volatile *N*-nitrosamines have been shown to be mutagenic in a battery of short-term test systems and appear to be carcinogenic in a number of animal species (*3*).

In conclusion, in our study, urinary excretion of *N*-nitrosamines was observed during high nitrate exposure. Moreover, an increase in the incidence of the genetic biomarker *HPRT* VF in peripheral lymphocytes was observed in subjects drinking water with high nitrate levels. We therefore conclude that drinking water contamination by nitrate implies a genetic risk. In a recent risk assessment study of the European Environmental Research Organization on nitrate, nitrite, and *N*-nitroso compounds, it was concluded that there is no firm evidence that dietary nitrate, derived principally from vegetables, constitutes a health hazard to humans (*3*). However, it might be that high nitrate intake derived from drinking water implies a greater risk than nitrate derived from vegetables due to the absence of protective factors like vitamin C. Adverse health effects of *N*-nitroso compounds should be considered in setting drinking water standards for nitrate. We intend to perform further molecular epidemiological studies on nitrate exposure using methylated DNA adducts in lymphocytes and target tissues as biomarkers of *N*-nitrosamine formation.

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